

SAM68 Regulates Neuronal Activity-Dependent Alternative Splicing of Neurexin-1

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SUMMARY

The assembly of synapses and neuronal circuits relies on an array of molecular recognition events and their modification by neuronal activity. Neurexins are a highly polymorphic family of synaptic receptors diversified by extensive alternative splicing. Neurexin variants exhibit distinct isoform-specific biochemical interactions and synapse assembly functions, but the mechanisms governing splice isoform choice are not understood. We demonstrate that *Nrxn1* alternative splicing is temporally and spatially controlled in the mouse brain. Neuronal activity triggers a shift in *Nrxn1* splice isoform choice via calcium/calmodulin-dependent kinase IV signaling. Activity-dependent alternative splicing of *Nrxn1* requires the KH-domain RNA-binding protein SAM68 that associates with RNA response elements in the *Nrxn1* pre-mRNA. Our findings uncover SAM68 as a key regulator of dynamic control of *Nrxn1* molecular diversity and activity-dependent alternative splicing in the central nervous system.

INTRODUCTION

Neuronal activity-dependent signaling pathways play a central role in circuit assembly and plasticity processes in the brain (Wong and Ghosh, 2002; Davis, 2006). Neurons modify their molecular repertoire in response to cellular depolarization through transcriptional programs and fine-tuning of protein turnover (Cohen and Greenberg, 2008; Bingol and Sheng, 2011). More recently, activity-dependent alternative splicing has emerged as an additional mechanism for the dynamic modification of neuronal function where production of specific splice variants modifies cellular trafficking, signaling properties or function of synaptic proteins (An and Grabowski, 2007; Li et al., 2007b).

Signaling pathways for activity-dependent splicing regulation are only beginning to be uncovered. Calcium/calmodulin-dependent kinase IV (CaMKIV) activation modifies alternative splicing in cultured neuronal cells. Some pre-mRNAs contain CaMKIV-responsive elements (CaRRE1 and 2) and UAGG motifs that impose depolarization-dependent regulation (Xie and Black, 2001; An and Grabowski, 2007; Lee et al., 2007). However, these RNA elements are not found in all pre-mRNAs subject to depolarization-dependent alternative splicing, and additional response elements remain to be uncovered.

One class of synaptic cell surface receptors that is extensively regulated at the level of alternative splicing is the Neurexins (Ushkaryov et al., 1992). Neurexins contribute to the assembly of functional presynaptic terminals (Dean et al., 2003; Missler et al., 2003; Li et al., 2007a), and mutations in the human neurexins are associated with neurodevelopmental disorders (Südhof, 2008; Rujescu et al., 2009). Three neurexin genes (*Nrxn1*, 2, 3) each encode two primary neurexin transcripts (*alpha* and *beta*) that are subject to alternative splicing at five positions yielding more than 3,000 protein-coding mRNAs (Rowen et al., 2002; Tabuchi and Südhof, 2002; Baudouin and Scheiffele, 2010). This molecular diversity as well as the capability of neurexins to organize synaptic structures led to the hypothesis that alternative splicing may give rise to cell-type- and/or synapse-specific functions (Chih et al., 2006; Craig and Kang, 2007; Huang and Scheiffele, 2008).

Most attention of functional studies has focused on the *Nrxn* alternatively spliced segment 4 (AS4), which is highly conserved in all *Nrxn* pre-mRNAs. Incorporation of exon 20 at AS4 generates NRX 4(+) protein variants containing a 30 amino acid insertion, whereas skipping results in the 4(−) variant. Importantly, alternative splicing of *Nrxns* may underlie an adhesive code: The NRX 4(+) and 4(−) variants exhibit differential interactions with several ligands that are critical mediators of synaptogenesis, including neuroligins, leucine-rich repeat proteins (LRRs), and the Cbln1-GluD2 complex (Ichtchenko et al., 1995; Boucard et al., 2005; Chih et al., 2006; de Wit et al., 2009; Koehnke et al., 2010; Siddiqui et al., 2010; Uemura et al., 2010; Matsuda and Yuzaki, 2011). For example, the NRX1 4(+) isoform exhibits only weak

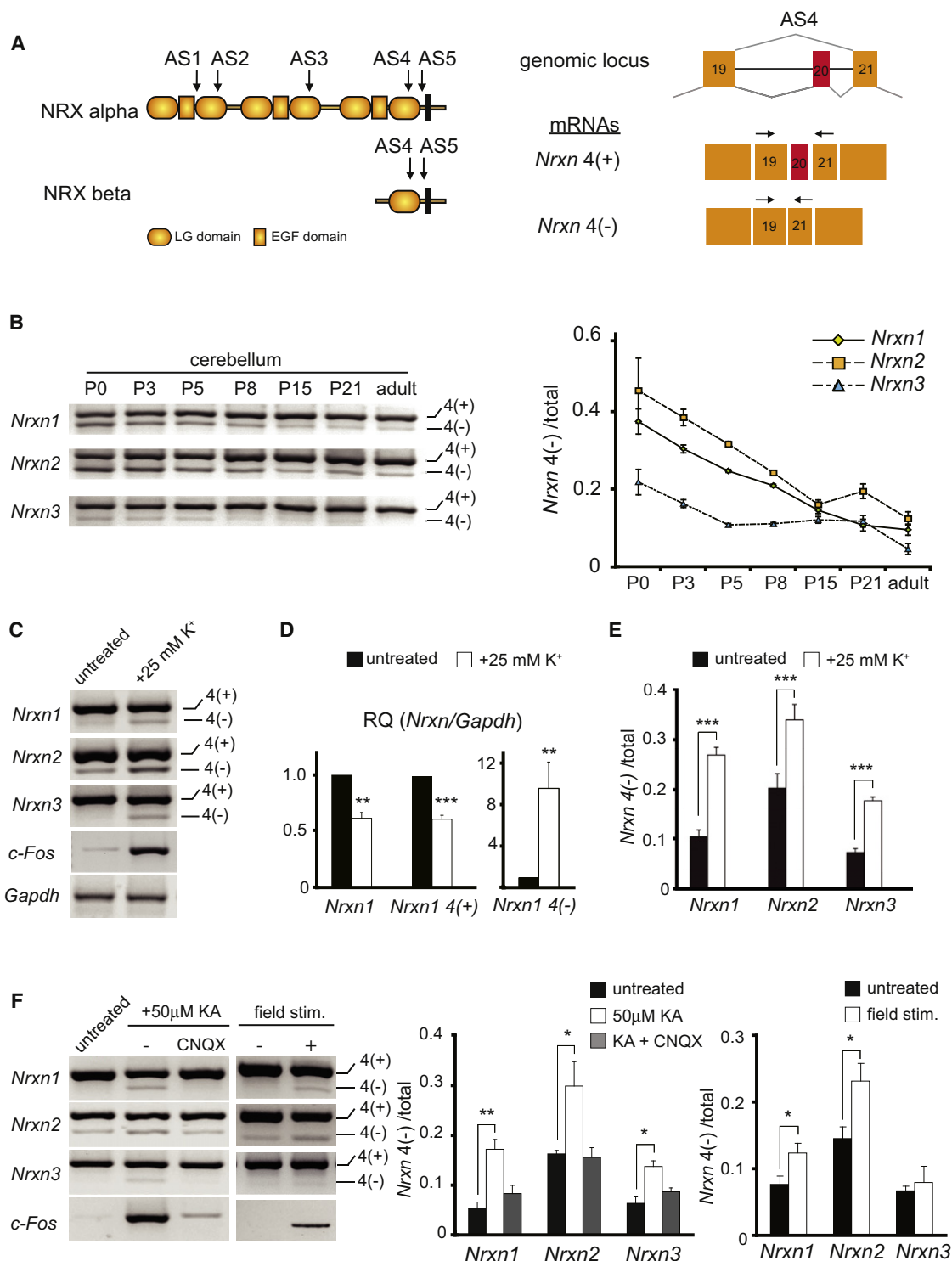


Figure 1. Depolarization-Dependent Alternative Splicing of Neurexins in Cerebellar Neurons

(A) Schematic diagram outlining organization of Neurexin alpha and neurexin beta protein variants. The positions of alternatively spliced segments (AS1,2,3,4,5) are indicated by arrows. LamininG domains are shown as ovals, EGF domains as rectangles, black line is the trans-membrane domain. The cartoon on the right illustrates the 90 bp cassette exon (20) at AS4. Position of primers used for analysis indicated by arrows.

(B) Developmental regulation of *Nrnx*s at AS4 in mouse cerebellum (n = 3).

(C) Dissociated cultures of mouse cerebellar neurons maintained in vitro for 14 days were depolarized by addition of 25 mM KCl for 6 hr. Semiquantitative PCR analysis with primers for AS4, *c-Fos*, or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

adhesion and binding with the neuroligin-1B postsynaptic receptor (NL1B), whereas the NRX1 4(–) isoform engages in strong adhesive interactions (Dean et al., 2003; Chih et al., 2006; Reissner et al., 2008). Therefore, alternative splicing at AS4 controls a fundamental switch in neurexin function at synapses.

The critical functional consequences of *Nrxn* alternative splicing at AS4 raise the question of how alternative splice isoform choice is regulated. Genome-wide identification of mRNA targets for several RNA-binding proteins has not yielded insights into *Nrxn1* AS4 regulation (Ule et al., 2005; Boutz et al., 2007). Other studies reported modifications of splicing patterns after long-term manipulation of neuronal activity and in response to fear conditioning (Rozic-Kotiroff and Zisapel, 2007; Kang et al., 2008; Rozic et al., 2011). However, the RNA response elements and RNA-binding proteins conferring alternative splice isoform choice in *Nrxns* have not been identified. In fact, the RNA-binding proteins controlling activity-dependent alternative splicing of any neuronal pre-mRNA are not understood. Here, we describe a signaling pathway and RNA response elements for *Nrxn1* and demonstrate that the KH-domain RNA-binding protein SAM68 (Src-associated in mitosis 68 kDa protein) is a key regulator of activity-dependent alternative splicing in the central nervous system.

RESULTS

Neurexin Alternative Splicing Is Regulated by Neuronal Activity

Incorporation of the cassette exon at AS4 (exon 20) in *Nrxn1*, 2, 3 varies across brain regions (Figure 1A; Figure S1 available online). Moreover, for some regions exon 20 inclusion differs between the three *Nrxns* indicating gene-specific regulation. In the developing cerebellar cortex *Nrxn1* 4(–) mRNAs declines successively from postnatal day 0 to postnatal day 21 (Figure 1B). This reveals spatial and developmental regulation of *Nrxn* alternative splicing in the mouse brain.

The changes in *Nrxn* splicing regulation during the first 2 postnatal weeks parallel the developmental shift in resting membrane potential of cerebellar granule cells (–25 to –55 mV) (Rossi et al., 1998). To test whether alternative splicing is altered by membrane depolarization we stimulated cultured granule cells by addition of 25 mM potassium chloride (KCl), which shifts the membrane potential to approximately –36 mV (Mellor et al., 1998) (Figures 1C–1E). After 6 hr, the abundance of *Nrxn1* 4(–) mRNA was increased nearly 10-fold as measured by quantitative PCR ($n = 4$, $p < 0.01$, Figure 1D). This was accompanied by a decrease in the *Nrxn1* 4(+) and total *Nrxn1* transcript indicating that in addition to the increase of *Nrxn1* 4(–) also the turnover of the preexisting *Nrxn1* mRNA might be altered by depolarization (Figure 1D). Using a semiquantitative PCR assay with flanking primers we found that transcripts from all three neurexin

genes (*Nrxn1*, 2, 3) each responded similarly to depolarization ($n = 4$, $p < 0.001$, Figures 1C and 1E). The shift in *Nrxn1* alternative splicing was detectable upon a depolarizing stimulus of 30 min and was reversible, as *Nrxn1* 4(–) levels returned to baseline 48 hr after stimulation (Figures S2A and S2B).

To explore whether alternative splicing was controlled by neuronal activity we stimulated glutamate receptors by application of kainic acid (50 μ M for 6 hr) or applied electrical field stimulation (3 \times 3 min at 200 Hz spaced by 7 min intervals). Both paradigms produced a significant increase in *Nrxn1* and *Nrxn2* 4(–) mRNA (for electrical stimulation $n \geq 7$, $p < 0.05$, Figure 1F). *Nrxn3* 4(–) was not significantly upregulated in response to electrical stimulation. The activity-dependent alternative splicing shift in *Nrxn1* AS4 was further confirmed by quantitative PCR (Rq *Nrxn1* 4(–)/*Gapdh* increased 2.4 ± 0.4 -fold compared to non-stimulated control, $n = 6$, $p = 0.004$). Importantly, depolarization did not significantly alter exon incorporation at the neighboring alternatively spliced segments 3 and 5 in *Nrxn1* (Figure S2C). This highlights site-selective activity-dependent splicing regulation for *Nrxn1* AS4 in cerebellar neurons.

We assessed the effects of pharmacological inhibitors to obtain insight into the signaling mechanisms underlying the regulation of *Nrxns* at AS4. Blockade of voltage-gated calcium channels by 20 μ M Cd^{2+} or the L-type voltage-gated calcium channel blocker nifedipine abolished the depolarization-dependent splicing shifts ($n = 4$, $p < 0.01$, Figure 2A). The depolarization-induced increase in the *Nrxn1* 4(–) isoform was abolished by application of the CaMK inhibitors KN-62 and KN-93 ($n = 5$, $p < 0.01$, Figure 2B). By contrast, inhibitors of the MAP-Kinases MEK1 and MEK2 (U0126) and calcineurin (FK506) had no significant effect on depolarization-dependent *Nrxn* splicing regulation (Figure S2D). When cells were treated with actinomycin D during stimulation no increase in the *Nrxn* 4(–) splice variant was observed, demonstrating a requirement for ongoing transcription (Figure S2D). KN-62 and KN-93 inhibit CaMKs but also affect other kinases and neuronal receptors (Wayman et al., 2008). Therefore, we further examined the involvement of the CaMK pathway by inhibiting CaMK kinases with STO-609 (Tokumitsu et al., 2002) or by RNA interference knockdown of CaMKIV, the dominant nuclear CaMK isoform in granule neurons (Ohmsted et al., 1989). Both treatments reduced depolarization-induced alternative splicing ($n \geq 7$, STO609: $p < 0.01$, siCaMKIV: $p = 0.02$, Figure 2B). These experiments uncover a depolarization-induced, calcium- and CaMKIV-dependent mechanism for alternative splicing of *Nrxn1*.

Depolarization-Dependent Alternative Splicing Alters Response to Synaptogenic Receptors

Alternative splicing of *Nrxns* at AS4 regulates adhesive interactions with synaptic receptors (Figure 2C). NRX1 4(–) but not

(D) Quantitative real-time PCR for total *Nrxn1*, *Nrxn1* 4(+), and *Nrxn1* 4(–). *Nrxn* mRNA/*Gapdh* values in untreated control cells were set to 1.0 and compared to cells stimulated (25 mM KCl) for 6 hr ($n = 4$).

(E) Analysis of semiquantitative PCR data from (C). The fraction of *Nrxn* 4(–) compared to total *Nrxn* is plotted ($n = 4$).

(F) The glutamate receptor agonist kainic acid (50 μ M KA) was applied for 6 hr in presence or absence of the AMPA/kainate receptor antagonist CNQX (50 μ M) ($n = 4$ –5 independent cultures). Electrical field stimulation was applied in three trains (3 min of 200 Hz) spaced by 7 min and RNA was isolated 5.5 hr after stimulation ($n = 6$ –8 independent cultures per condition).

Mean and SEM. See also Figures S1 and S2.

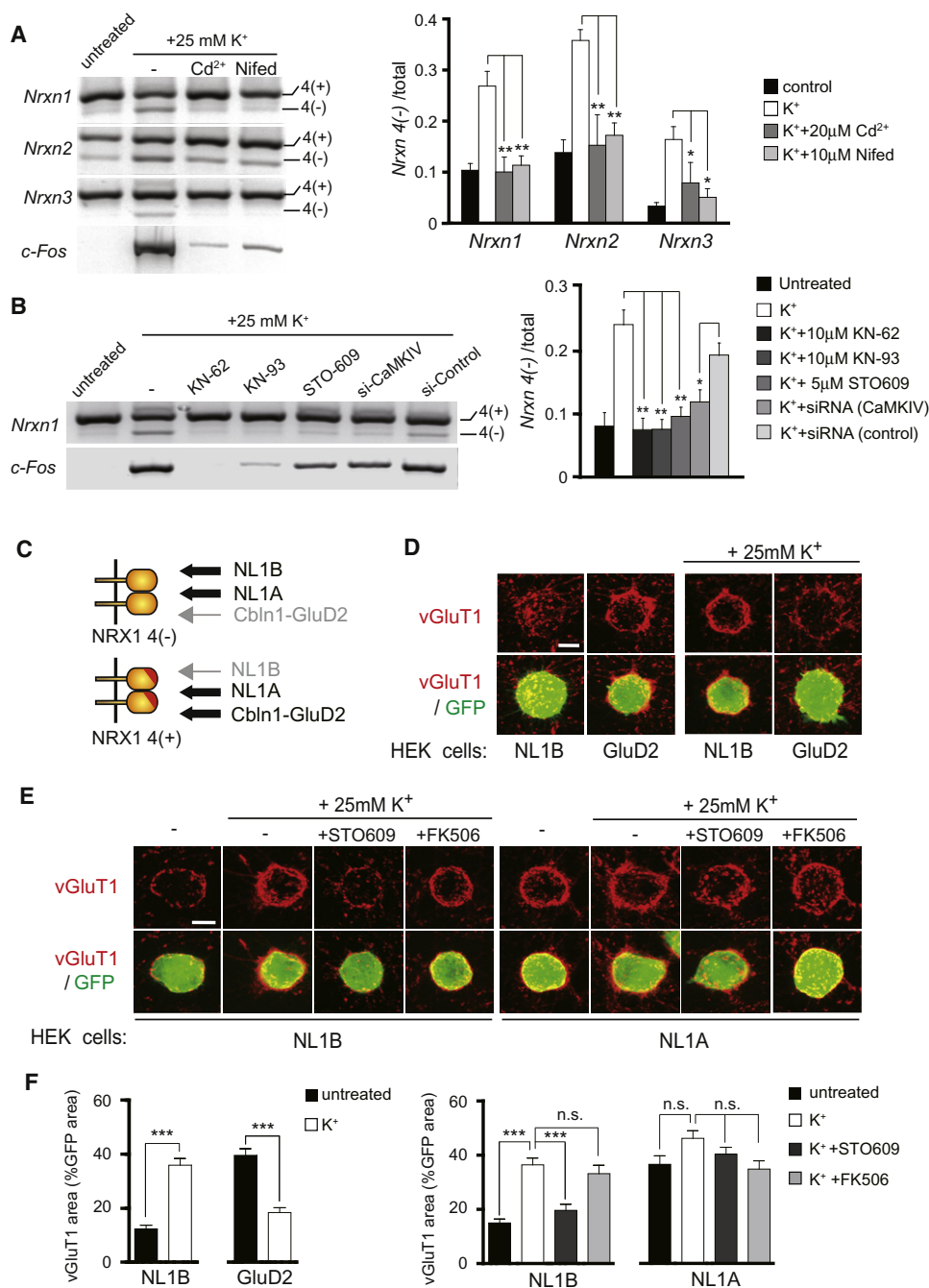


Figure 2. Depolarization-Dependent Alternative Splicing of Neurexin Requires CaMKIV and Controls *trans*-Synaptic Signaling

(A) Dissociated cultures of mouse cerebellar neurons 14 days in vitro (DIV) were depolarized (25 mM KCl) for 6 hr and CdCl₂ (20 μM) or nifedipine (10 μM) were added as indicated. *Nrnx* 4(-) levels were quantified by semiquantitative PCR (n = 4).

(B) CaMK signaling in depolarization-dependent alternative splicing of *Nrnx1* was inhibited by coapplication of pharmacological inhibitors or RNAi knockdown of CaMKIV (n = 4–7). c-Fos activation was probed by RT-PCR to confirm immediate-early gene induction.

(C) Schematic diagram illustrating binding preferences of neurexin splice isoforms. Beta-NRX1 4(-) interacts similarly with the NL1A and NL1B splice variants, the 4(+) insertion in the beta-NRX1 4(+) variant (red insert) prevents strong adhesive interactions with NL1B but still allows for interactions with NL1A. The Cbln1/GluD2 complex specifically interacts with the 4(+) insertion in beta-NRX1 4(+).

(D) HEK293 cells coexpressing GFP and NL1B (left) or GluD2 (right) were added to cerebellar neurons at DIV9 and analyzed 2 days later. For depolarizing conditions, 25 mM KCl was added to the culture medium at DIV 6. Presynaptic differentiation assessed by vGluT1 staining.

NRX1 4(+) forms strong *trans*-synaptic adhesion complexes with the neuroligin-1B receptor (NL1B) (Dean et al., 2003; Boucard et al., 2005; Chih et al., 2006). Conversely, NRX1 4(+) but not NRX1 4(−) binds the synaptic Cbln1-GluD2 complex (Uemura et al., 2010; Matsuda and Yuzaki, 2011). To test whether the depolarization-induced alternative splicing switch in *Nrxns* translates into altered *trans*-synaptic signaling we employed a coculture system where primary neurons are combined with non-neuronal cells expressing a single postsynaptic receptor isoform (Scheiffele et al., 2000). NL1B-expressing HEK293 cells induced only low levels of presynaptic differentiation as measured by the accumulation of the synaptic vesicle marker vGluT1 (Figure 2D). By contrast, GluD2-expressing cells triggered robust recruitment of this marker. These findings are consistent with weak functional coupling between NL1B and NRX1 4(+), the primary *Nrxn1* isoform expressed in unstimulated granule cells, and strong coupling of NRX1 4(+) to the Cbln1-GluD2 complex. When granule cells were maintained under depolarizing conditions, the response to NL1B-expressing cells was strongly increased and responses to GluD2 were reduced (Figures 2D, 2F, and S2E). Interestingly, this reduction in GluD2 responses is likely due to both, a change in *Nrxn1* alternative splicing but also reduced expression of Cbln1, a critical component of the tripartite NRX-Cbln1-GluD2 complex (Figure S2E) (Matsuda et al., 2010; Iijima et al., 2009).

We further examined the mechanisms of altered NL1B responses in this assay. Inhibition of the CaMK pathway by addition of STO609 suppressed not only *Nrxn1* 4(−) production but also depolarization-induced responsiveness to NL1B ($n = 35$ cells per condition, $p < 0.001$, Figures 2E and 2F). By contrast, inhibition of calcineurin, another calcium-dependent signaling molecule activated in depolarized granule cells had no effect on NL1B-induced presynaptic differentiation (FK506, Figures 2E and 2F). To confirm that the altered presynaptic assembly in depolarized cells was indeed due to neurexin-receptor binding specificities we compared the induction of presynaptic structures with the NL1A isoform which exhibits similar adhesion and binding affinities for both, NRX1 4(+) and NRX1 4(−) (Boucard et al., 2005; Chih et al., 2006; Koehnke et al., 2010). Presynaptic differentiation induced by NL1A did not differ significantly between nondepolarized and depolarized granule cells ($n = 37$ cells per condition, $p = 0.29$, Figures 2E and 2F), highlighting that the depolarization-induced increase in presynaptic assembly in response to NL1 is dependent on the identity of the postsynaptic neuroligin receptor isoform.

SAM68 Regulates *Nrxn1* AS4 Alternative Splicing In Vitro

To identify RNA-binding proteins that regulate *Nrxn1* alternative splicing, we established an assay in heterologous cells. A splice reporter spanning *Nrxn1* AS4 (*Nrxn1*-4, exons 19–21, Figure 3A) primarily produced the *Nrxn1* 4(+) splice isoform when trans-

ected into HEK293 cells, therefore providing an assay to identify proteins that promote exon 20 skipping. A survey of the intronic sequences flanking exon 20 revealed AU-rich sequence elements reminiscent of binding sites for the RNA-binding protein SAM68 (Lin et al., 1997; Galarneau and Richard, 2009). Therefore, we examined whether SAM68 could regulate alternative splicing of the *Nrxn1*-4 reporter. In addition, we tested several other RNA-binding proteins implicated in neuronal alternative splicing (Nova-1, Nova-2, nPTB, hnRNPA1, hnRNPH) (Matter et al., 2002; Ule et al., 2005; Chawla et al., 2009; Fiset et al., 2010). Of these proteins examined, only SAM68 effectively shifted the reporter splicing pattern to produce *Nrxn1* 4(−) ($n = 3$, $p < 0.001$, Figure 3B). SAM68 is a member of the STAR (“signal transduction and activation of RNA”) family of RNA-binding proteins containing a single KH domain (Volk et al., 2008). SLM1 and SLM2 are two SAM68-like mammalian proteins, which share 70%–80% amino acid sequence identity with SAM68 in their RNA-binding domains (Di Fruscio et al., 1999). Both, SLM1 and SLM2 exhibited similar activities as SAM68 in shifting alternative splicing to the 4(−) isoform (Figure 3B). SAM68 function in this assay was dose dependent as increasing amounts of SAM68 resulted in increased exclusion of exon 20 (Figure 3C).

SAM68 has been characterized as a nuclear RNA-binding protein but also non-nuclear functions have been described (Grange et al., 2004; Henao-Mejia et al., 2009; Huot et al., 2009). SAM68 point mutants (I184N and G178E) that abolish RNA binding (Lin et al., 1997; Chawla et al., 2009) or nuclear targeting (Y440F) had strongly reduced function in the splice reporter assay (Figure 3D). Similarly, SAM68 function was inhibited when SAM68 was cotransfected with constitutively active Fyn kinase (Figure 3E), which inactivates the nuclear-localization signal of SAM68 (Paronetto et al., 2007). Thus, SAM68-mediated splicing regulation of *Nrxn1* AS4 relies on RNA binding and SAM68 transport to the nucleus.

Nrxn1 Contains Multiple Intronic SAM68 Response Elements

We sought to identify the RNA elements which control SAM68-dependent alternative splicing of *Nrxn1*. Deletion analysis in intron 19 and intron 20 identified sequences in both introns that significantly reduce SAM68-induced skipping of exon 20 when deleted from the reporter construct (Figures 4A and S3). A maximum reduction in SAM68-dependent splicing shifts was obtained with deletions 19-c and 20-c, respectively (Figure 4A). When deletion 19-c and deletions in intron 20 were combined (constructs 19/20-a,b,c,d), SAM68-induced exon skipping was completely abolished for constructs 19/20-c and 19/20-d ($n = 4$, $p < 0.001$, Figure 4A). This suggests that multiple intronic SAM68 response elements located in introns 19 and 20 cooperate in regulating *Nrxn1* AS4. Notably, the apparent SAM68 response element between deletion sites 20-b and 20-c contains multiple AU-rich sequence elements conforming to SAM68

(E) HEK293 cells coexpressing GFP and NL1B (left) or NL1A (right) were added at DIV7 under depolarizing or control conditions. Pharmacological inhibitors (5 μ M STO609 or 10 μ M FK506) were applied at DIV 6.

(F) Morphometric analysis of vGluT1 staining at neuron-HEK293 contact sites analyzing the fraction of the vGluT1-positive area of the GFP-positive neuroligin-expressing cell ($n > 30$ cells per condition, three independent experiments).

Mean and SEM. Scale bars are 5 μ m. See also Figure S2.

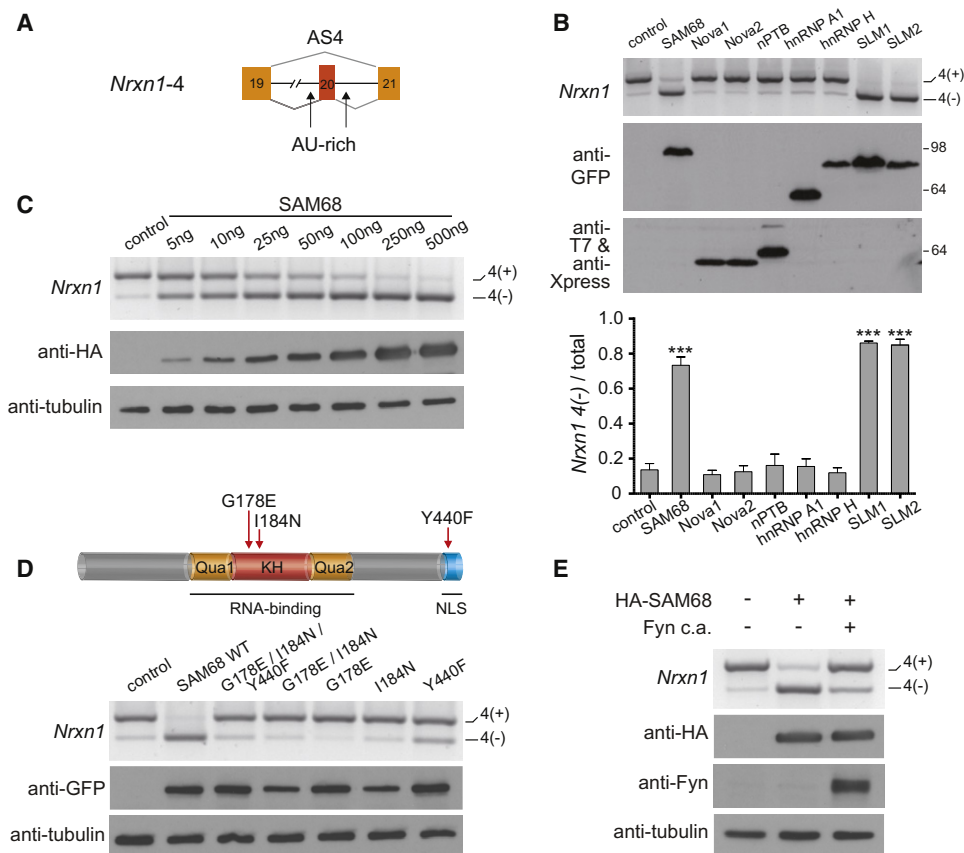


Figure 3. SAM68-Dependent Alternative Splicing of Neurexins In Vitro

(A) Schematic diagram of *Nrnx1-4* splice reporter construct containing AS4 with constitutive exons (orange), alternative exon 20 (red), and introns shown as lines. Two AU-rich sequence stretches are highlighted with arrows. Intron 19 (>40 kbp) was truncated leaving >550 bp adjacent to the splice donor and the acceptor sites intact.

(B) Splice reporter expression vector was cotransfected into HEK293 cells with negative control DNA or epitope-tagged expression constructs for GFP-SAM68, T7-Nova1, T7-Nova2, Xpress-nPTB, YFP-hnRNPA1, YFP-hnRNPH, GFP-SLM1, GFP-SLM2. Alternative splice isoform choice was measured by semi-quantitative RT-PCR with primers flanking the alternative splice site (n = 3). Expression of all transfected RNA-binding proteins was confirmed by western blotting with anti-GFP, -T7, or -Xpress antibodies. Molecular weights in kDa.

(C) Cotransfection of increasing amounts of a SAM68 expression vector with the *Nrnx1-4* splice reporter. Alternative splicing was assayed by semiquantitative RT-PCR and HA-tagged SAM68 detected by immunoblot.

(D) GFP-tagged point mutants in SAM68 KH domain and nuclear localization signal were coexpressed with the *Nrnx1-4* splice reporter and alternative splicing was analyzed by semiquantitative RT-PCR. Protein expression levels were compared using western blotting.

(E) Cotransfection of plasmids encoding constitutively active Fyn-kinase and *Nrnx1-4* reporter.

Mean and SEM.

RNA-binding sites in other pre-mRNAs (Lin et al., 1997; Galarneau and Richard, 2009). Therefore, we tested binding of SAM68 to synthetic RNA-oligonucleotides (probes A and B) each spanning 30 bases of the presumptive binding region (Figure 4B). In pull-down assays, probe A yielded efficient binding of endogenous SAM68 from brain extracts, whereas only background binding was observed for probe B (Figure 4B). SLM1 and SLM2 exhibited similar interaction with probe A as SAM68. Importantly, under the same conditions, SF2 and NeuN/Fox3, two other RNA-binding proteins (Kim et al., 2009) were not recovered in the precipitates. Mutation of two nucleotides in a presumptive SAM68-binding site of probe A (probe A*) (Figure 4B) significantly reduced the recovery of SAM68 in the pull-down experiment (Figure 4B). Therefore, in heterologous cells,

SAM68 promotes skipping of exon 20 through association with intronic SAM68 response elements in the *Nrnx1* pre-mRNA.

Next, we developed an in vivo RNA response element assay (iRRE) to test whether the SAM68 response elements are essential for *Nrnx1* AS4 splicing regulation in the intact nervous system. We delivered the *Nrnx1-4* reporters specifically into mouse cortical pyramidal cells in vivo by in utero electroporation followed by analysis at postnatal day 6 (P6). Histochemical and semiquantitative PCR analysis of cortical sections confirmed similar levels of mutant and wild-type (WT) splice reporter expression in cortical layer 2/3 pyramidal cells (Figure 4C). Alternative splicing of the *Nrnx1-4* reporter was comparable to the products detected for endogenous *Nrnx1* in total cortical RNA samples (compare Figure S1). Notably, deletion of the SAM68

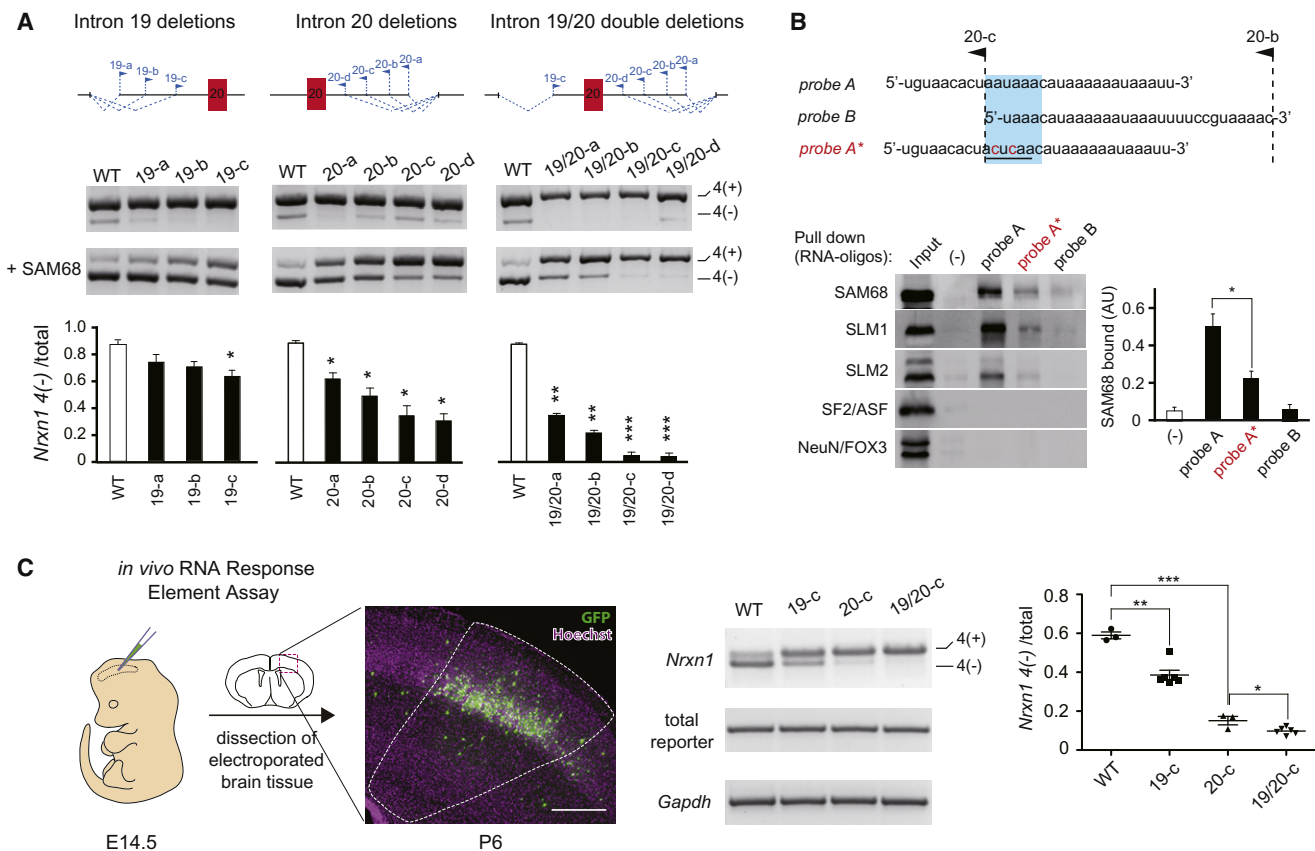


Figure 4. Mapping of SAM68 Response Elements in *Nrxn1*

(A) Intronic sequences preceding (19-a, 19-b, 19-c) or following exon 20 (20-a to 20-d) were deleted from *Nrxn1-4*. Intron 19-c deletion was combined with deletions 20-a to 20-d in intron 20. Panels show semiquantitative PCR results from HEK293 cells without and with coexpression of SAM68 and *Nrxn1-4*, respectively. Quantitation was performed for products in presence of SAM68 ($n = 4$).

(B) Biotinylated RNA-oligonucleotide probes covering intronic sequence between 20-b and 20-c deletions used in pull-down experiments with mouse adult brain extracts. Probe A* contains two nucleotide changes (red). Bound proteins were analyzed by western blotting with anti-SAM68, anti-SLM1, anti-SLM2, anti-Fox3/NeuN, and anti-SF2/ASF antibodies. SAM68 binding was quantified by densitometric scanning of western blot signals ($n = 4$).

(C) Splice reporters were delivered into cortical pyramidal cell precursors of E14.5 embryos by in utero electroporation and reporter processing was examined at P6. Reporter expression levels were examined with primers specific for constitutive reporter sequence elements and RNA input levels were monitored by semiquantitative PCR for endogenous *Gapdh* ($n = 3-6$). Scale bar is 500 μm . Mean and SEM. See also Figure S3.

response elements abolished *Nrxn1* 4(-) production in the iRRE assay ($n = 3$ to 6 animals per construct, $p < 0.01$ and 0.001 for 19-c and 20-c, respectively; Figure 4C). This demonstrates that the intronic response elements are essential for *Nrxn1* 4(-) production in the context of the endogenous splicing machinery in cortical pyramidal cells in vivo.

Cell-Type-Specific Expression of STAR Proteins in the Mouse Cerebellum

To begin to examine whether endogenous STAR proteins are critical regulators of *Nrxn1* alternative splicing in vivo we examined SAM68, SLM1, and SLM2 expression patterns. Affinity-purified antibodies specifically recognized the respective STAR family proteins in western blots and identified significant expression across various brain areas (Figures 5A and 5B). In the developing cerebellum SAM68 immune-reactivity was high at birth and slightly declined over the first 3 weeks. SLM1 immune-

reactivity increased over the first 3 postnatal weeks, whereas SLM2 protein levels decreased (Figure 5C). Notably, SAM68, SLM1, and SLM2 differed significantly in their cellular expression patterns in the cerebellar cortex (Figure 5D). SAM68 was detected in most neuronal cell populations with high immune-reactivity in cerebellar granule cells of the internal granular layer (IGL), ROR α -positive Purkinje cells, as well as IGL and molecular layer interneurons. SLM1 immunoreactivity was predominantly observed in Purkinje cells, whereas SLM2 antibodies stained selectively IGL and (less significantly) molecular layer interneurons (Figure 5D). Considering that of all STAR family proteins only SAM68 was significantly expressed in cerebellar granule cells (the major source of cerebellar mRNA), we further examined its distribution during development. At P7, SAM68 was highly expressed in granule cell precursor cells in the EGL (external granular layer) and mature granule cells of the IGL (Figure 5E). Therefore, SAM68 is well positioned to regulate *Nrxn1* alternative

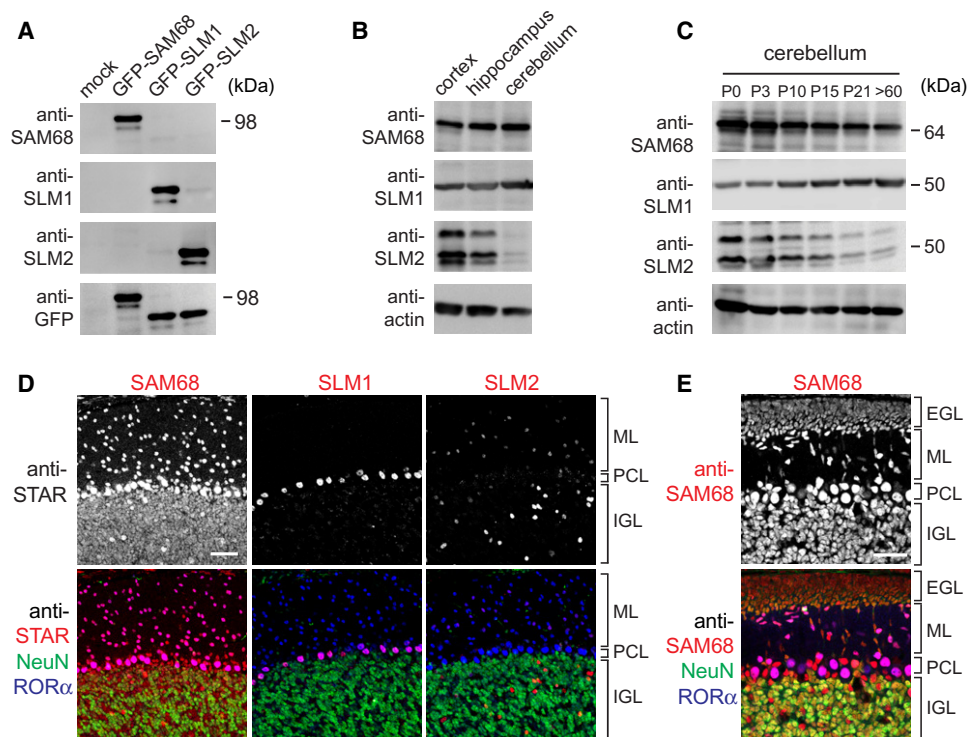


Figure 5. Cell-Type-Specific STAR Protein Expression in the Cerebellum

(A) Specificity of anti-STAR protein antibodies confirmed by probing lysates from HEK293 cells overexpressing GFP-tagged SAM68, SLM1, or SLM2.

(B) Immunoblot of adult cortical, hippocampal, and cerebellar tissue extracts probed with STAR protein antibodies.

(C) Developmental regulation of STAR protein expression in the cerebellum from postnatal day 0 (P0) to adult (>P60).

(D and E) Parasagittal sections through adult cerebellum triple stained with anti-NeuN, anti-ROR α , and anti-SAM68, anti-SLM1, or anti-SLM2 antibodies. In (E), P7 cerebellum was analyzed using anti-SAM68 antibodies. ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granular layer; EGL, external granular layer. Scale bars are 50 μ m. See also Figure S4.

splicing in granule cells during development and in the adult cerebellum.

SAM68 Regulates *Nrxn1* Alternative Splicing In Vivo

To explore how loss of SAM68 affects neuronal alternative splicing regulation, we examined SAM68 knockout (KO) mice (Richard et al., 2005). In the mutant mice, SAM68 protein was undetectable, whereas expression of SLM1 and SLM2 was not significantly altered (Figures 6A and S4A). Notably, SAM68 KO mice exhibit defects in basal motor performance (Lukong and Richard, 2008). To further assess a potential impairment of cerebellar function in SAM68 KO mice we tested their motor learning on an accelerating rotarod. SAM68 KO mice performed significantly worse than WT mice ($p < 0.01$). Behavioral defects persisted over a 7 day training period demonstrating that KO mice have significant motor coordination deficits (Figure 6B).

SAM68 KO cerebella do not show gross anatomical alterations as judged by histology (Figure 6A) and quantitative western blotting for a panel of synaptic markers (Figure S4B). However, synaptic glomeruli formed by mossy fiber axons showed an abnormal accumulation of the presynaptic vesicle marker vGluT1 (Figures 6C and 6D). Quantitative analysis of vGluT1 staining intensity revealed a shift in the distribution of mossy fiber

rosettes toward higher staining intensities ($n > 438$ rosettes per genotype, $p < 0.05$, Kolmogorov-Smirnov test, Figure 6D). By contrast, staining intensity for the presynaptic marker VAMP2 was unaltered ($p > 0.1$, Kolmogorov-Smirnov test, Figure 6D). Consistent with a significantly shifted distribution also the mean staining intensity for vGluT1 within mossy fiber rosettes was significantly increased in SAM68 KO cerebellum ($31\% \pm 7\%$ increase in the KO, $n = 5$ animals per genotype, $p < 0.001$). Although the exact molecular and anatomical underpinnings of this synaptic phenotype remain to be defined, these findings indicate that SAM68 KO mice exhibit alterations in cerebellar mossy fiber synapses in vivo.

Alternative splicing of *Nrxn1* at AS4 in SAM68 KO mice was severely perturbed. The abundance of the *Nrxn1* 4(−) form in the cerebellum was reduced fourfold at P7, a developmental stage where WT cerebellar cells express higher levels of *Nrxn1* 4(−) and 3-fold in the adult ($n = 3$, $p < 0.001$, Figure 6E). Importantly, the overall level of *Nrxn1* mRNA was not altered indicating a change in alternative splicing rather than mRNA stability. Analysis of brainstem and cortical mRNAs confirmed a similar reduction in *Nrxn1* 4(−) and a corresponding increase in *Nrxn1* 4(+) ($n = 3$, $p < 0.001$, Figure 6E). There is accumulating evidence for coupling between transcription and splicing regulation resulting in promoter-specific splicing patterns (Batsché et al., 2006).

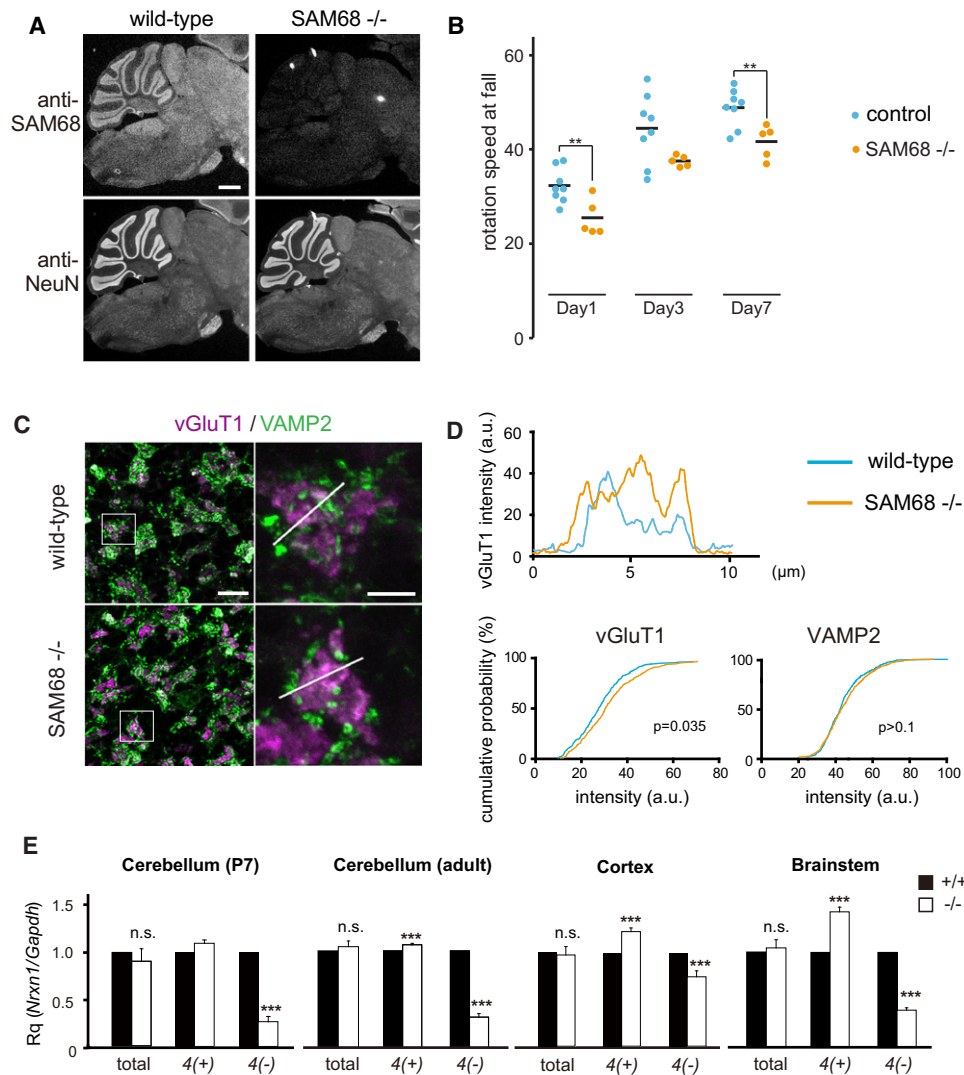


Figure 6. Cerebellar Alterations in SAM68 KO Mice

(A) Low magnification view of parasagittal section of brain stem and cerebellum reveals anti-SAM68 immune-reactivity that is lost in SAM68 KO ($-/-$) tissue. (B) Motor performance of male control mice and SAM68 KO littermates were tested on an accelerating rotarod. The latency to fall is plotted for days 1, 3, and 7 of training ($n = 5-8$ mice per genotype). (C) Immunoreactivity for synaptic vesicle proteins vGluT1 (magenta) and VAMP2 (green) in the internal granular layer (IGL) of adult WT and SAM68 KO cerebellar cortex. The right panel shows single rosettes at high magnification. Scale bars are 20 μm in left and 5 μm in right. (D) Representative line scans from WT and SAM68 KO cerebellum displaying immunoreactivity for vGluT1 in mossy fiber rosettes in (C). Cumulative probability of vGluT1 and VAMP2 average intensities in cerebellar glomeruli compared between WT (blue line) and SAM68 KO mice (orange) ($n > 438$ rosettes from three animals per genotype, Kolmogorov-Smirnov test). (E) Quantitative PCR performed on cortical, cerebellar, brainstem cDNAs from control (+/+; black) and SAM68 KO mice ($-/-$; white; $n = 3$ animals per genotype). Abundance of total *Nrxn1* mRNA, *Nrxn1* 4(+), and *Nrxn1* 4(-) transcripts is expressed compared to *Gapdh* (values for control tissue were set to 1.0). Mean and SEM. See also Figure S5.

However, the reduction in the *Nrxn1* 4(-) splice variant was detected for both, the *Nrxn1* α and β transcripts (Figure S4C). The splice isoform choice in the neighboring *AS3* or alternative splicing of *Nlgn1* and *Nlgn2*, which encode NRX1-binding partners, was unaltered in the SAM68 KO tissues analyzed (Figure S4C). Therefore, SAM68 is an essential regulator of *Nrxn1* alternative splicing in vivo.

SAM68 Controls Activity-Dependent Splicing of *Nrxn1*

To explore whether SAM68 regulates activity-dependent *Nrxn1* alternative splicing, we first assessed whether SAM68 is modified by neuronal depolarization. We did not observe any significant increase in SAM68 transcript, protein level, or change in subcellular localization in response to neuronal depolarization (Figure S5). Therefore, we explored posttranslational

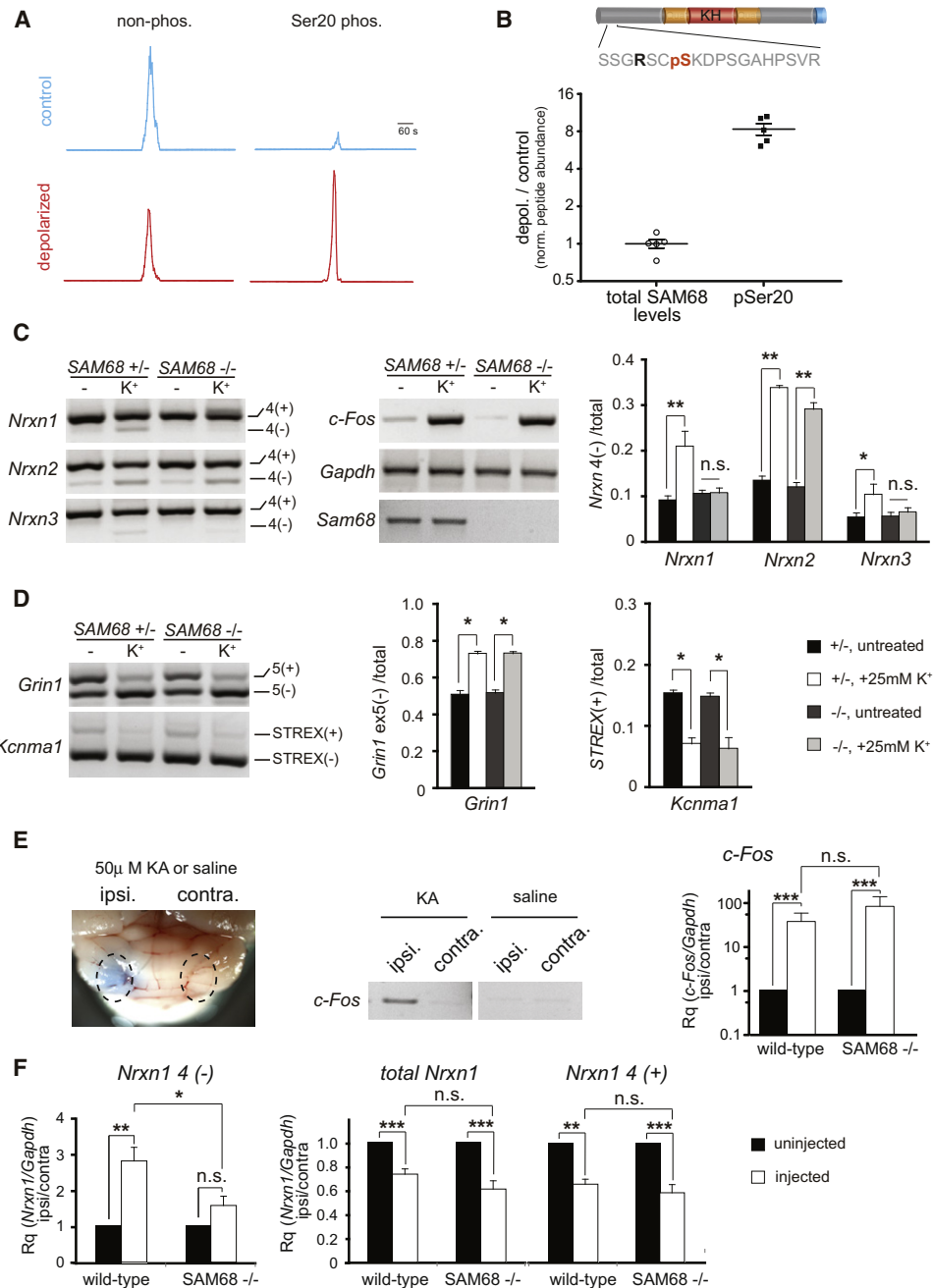


Figure 7. SAM68 Is Essential for Regulation of Activity-Dependent Splicing of *Nrnx1*

(A) Relative abundances of phosphorylated and nonphosphorylated SAM68 were assessed by liquid-chromatography-tandem mass spectrometry following SAM68-IP from control and depolarized cerebellar granule cells in culture.

(B) The CaMK consensus motif in the amino acid sequence surrounding Ser20 is highlighted in bold. Ratios of the normalized peptide abundances (depolarized/control) are shown (total SAM68 levels: 1.01 ± 0.08 , $p > 0.9$; phosphoserine 20: 8.39 ± 0.90 , $p < 0.01$).

(C) Heterozygous control (+/-) or homozygous SAM68 KO neurons (-/-) were incubated in depolarizing medium for 6 hr and exon 20 inclusion was monitored by semiquantitative PCR with flanking primers for *Nrnx1*, *Nrnx2*, or *Nrnx3* ($n = 4$). Loss of *Sam68* expression was confirmed with oligonucleotides amplifying the *Sam68* cDNA.

(D) Depolarization-dependent alternative splicing of the CaMK IV-responsive RNA element (CaRRE) containing pre-mRNAs *Grin1* exon 5 and *Kcnma1* STREX exon in control and SAM68 KO neurons ($n = 4$).

(E) Neuronal activity-dependent alternative splicing in vivo examined by stereotaxic injection of 50 μ M kainic acid (or saline as control) into the mouse cerebellum. Animals were sacrificed 5 hr after injection. Neuronal stimulation was confirmed by the increase in the expression of *c-Fos* mRNA in the injected ipsilateral versus noninjected contralateral side by semiquantitative (middle panel) and quantitative PCR (right panel, $Rq(c-Fos/Gapdh)$ for contralateral side set to 1.0, log scale, $n = 3$ animals).

modifications of SAM68. We analyzed SAM68 immunoprecipitated from control and depolarized cerebellar granule cells by tandem mass spectrometry (Figure 7A). A phosphopeptide modified at serine 20 was 8-fold enriched in the depolarized sample, whereas total SAM68 levels were unchanged ($n = 5$, $p < 0.01$; Figure 7B). Notably, serine 20 conforms to the consensus for CaMK-mediated phosphorylation (R-X-X-S/T) (Songyang et al., 1996) consistent with the regulation of SAM68 downstream of the CaMK pathway in response to neuronal activity.

To test whether SAM68 is required for depolarization-dependent alternative splicing regulation in cerebellar granule cells, cultures from control (SAM68^{+/+}) and KO mice (SAM68^{-/-}) were stimulated followed by transcript analysis. Control neurons (SAM68^{+/+}) exhibited a significant upregulation of the *Nrxn1* 4(-) splice variant but in SAM68^{-/-} neurons the depolarization-dependent alternative splicing shift at *Nrxn1* and *Nrxn3* AS4 was impaired ($n = 4$ per genotype $p < 0.01$, Figure 7C). Loss of depolarization-dependent alternative splicing was further confirmed by quantitative PCR for *Nrxn1*, which revealed a 7.4-fold upregulation in SAM68^{+/+} cells but no significant difference in SAM68^{-/-} cultures (RQ *Nrxn1* 4(-)/*Gapdh*: 7.4 ± 3.1 in +/– versus 1.05 ± 0.06 in –/–, $n = 4$ per genotype, $p < 0.02$). *c-Fos* upregulation was similar in control and homozygous KO neurons. Interestingly, depolarization-dependent alternative splicing of *Nrxn2* AS4 remained intact in SAM68 KO neurons (Figure 7C). Moreover, *Nrxn2* 4(-) splice variant abundance was only very modestly altered in SAM68 KO brains (Figure S6). These findings point to unexpected gene-specific mechanisms for alternative splicing regulation of *Nrxn1* and *Nrxn2* at AS4 in vivo.

We further explored the importance of SAM68 for activity-dependent alternative splicing programs by monitoring the CaRRE-containing pre-mRNAs *Grin1* exon 5 and the STREX exon of *Kcnma1*. For both pre-mRNAs, activity-dependent splicing was unaltered in SAM68 KO cerebellar neurons (Figure 7D). Therefore, SAM68 is essential for a specific subprogram of activity-dependent alternative splicing that includes regulation of *Nrxn1* but not *Grin1* or *Kcnma1* CaRRE-containing mRNAs.

Finally, we examined whether SAM68 is required for activity-dependent alternative splicing of *Nrxn1* AS4 in vivo. We stimulated cerebellar neurons by injection of the glutamate receptor agonist kainic acid directly into one cerebellar hemisphere of adult WT and SAM68 KO mice. Five hours after injection, animals were sacrificed and tissue was isolated from the injection site and the corresponding area of the uninjected hemisphere (Figure 7E). Transcript levels confirmed *c-Fos* induction in the injected hemispheres (Figure 7E). We assessed stimulation-induced alternative splicing of *Nrxn1* at AS4 by comparison of the injected versus noninjected hemisphere using quantitative PCR (Figure 7F). *Nrxn1* 4(-) transcripts were significantly increased in the stimulated hemisphere, whereas 4(+) containing transcripts were decreased ($n = 5$ animals, $p = 0.002$ for *Nrxn1* 4(-) and $p = 0.003$ for *Nrxn1* 4(+)). Interestingly, total *Nrxn1*

mRNA transcripts were reduced indicating that *Nrxn1* 4(+) mRNA destabilization may occur simultaneously with the activity-dependent increase of *Nrxn1* 4(-) production. In injected SAM68 KO cerebellum, the *Nrxn1* 4(-) upregulation was strongly attenuated (Figure 7F). The same KO samples showed normal *c-Fos* induction confirming successful KA injection and immediate early gene induction (Figure 7E). In summary, these findings demonstrate that SAM68 is required for activity-dependent alternative splicing of *Nrxn1* AS4 in vivo.

DISCUSSION

Alternative splicing of neuronal pre-mRNAs has emerged as an important mechanism for the functional diversification and dynamic regulation of neuronal proteins (Wojtowitz et al., 2004; Ule et al., 2005; Zhang et al., 2008). For neurexins, alternative splicing of AS4 represents a key mechanism for the regulation of NRX-ligand interactions. Our analysis identifies the KH-domain RNA-binding protein SAM68 as a critical regulator of this splice isoform choice that is essential for the activity-dependent regulation of *Nrxn1* in cerebellar neurons.

Alternative Splicing-Dependent Regulation of Neurexin Function

Alternative splicing of *Nrxn1* AS4 controls multiple splice-isoform-specific ligand interactions, including the binding to NL1B, Cbln1, and LRRTM1 and 2. Our results demonstrate that the depolarization-dependent *Nrxn* isoform switch upregulates *trans*-synaptic signaling via NL1B and at the same time, reduces the response to GluD2, which interacts through Cbln1 with NRX 4(+) variants (Uemura et al., 2010). Notably, *Cbln1* expression in granule cells is itself downregulated by neuronal activity (Iijima et al., 2009), and the reduction in Cbln1 levels also contributes to the loss of GluD2 responses. Therefore, depolarization-dependent alternative splicing of *Nrxn1* and downregulation of Cbln1 synergize to mediate a *trans*-synaptic ligand switch from interactions with GluD2 toward NL1B.

LRRTM proteins, a third group of NRX ligands, lack high affinity interactions with the NRX 4(+) variants and bind exclusively to the NRX 4(-) isoforms (Siddiqui et al., 2010). In SAM68 KO mice the *Nrxn1* 4(-) isoform is significantly reduced and our morphological analysis of SAM68 KO mice reveals abnormal distribution of the presynaptic marker vGluT1 in mossy fiber glomeruli, similar to a phenotype reported for *Lrrtm1* KO mice (Linhoff et al., 2009). Further studies will be required to elucidate whether the synaptic phenotype in SAM68 KO mice is due to a change in *Nrxn1* alternative splicing. However, our work provides a first indication for synaptic abnormalities caused by the loss of SAM68 in vivo.

Regulators of *Nrxn* AS4 Alternative Splicing

Cell- and tissue-specific alternative splicing are thought to be dependent on differentially expressed splicing factors or

(F) Inclusion of exon 20 in *Nrxn1* mRNA probed using quantitative PCR on cDNA derived from the injected cerebella ($n = 5$ animals per genotype). Rq(*Nrxn1*/*Gapdh*) values for the ipsilateral (injected) hemisphere (white columns) are expressed relative to the Rq(*Nrxn1*/*Gapdh*) values obtained for the uninjected contralateral side from the same animals (black columns).

Mean and SEM. See also Figure S6.

ubiquitous factors at different concentrations or activity (Chen and Manley, 2009). SAM68 is detected in most neuronal cells of the mouse cerebellum and throughout the nervous system (Figure 5; data not shown). Therefore, presence of SAM68 in a cell population is not predictive of the outcome of alternative splicing regulation of *Nrxn1* but SAM68 activity depends on neuronal depolarization (or additional stimuli). Resnick and colleagues implicated neuronal polypyrimidine tract-binding protein (nPTB) in the regulation of *Nrxn2* alternative splicing in cell lines (Resnick et al., 2008). Notably, our results indicate that the requirements for activity-dependent alternative splicing at AS4 differ between the *Nrxn1* and *Nrxn2* genes. *Nrxn2* alternative splicing at AS4 is not altered in most SAM68 KO neurons (Figure S6) and we did not detect an activity of nPTB toward *Nrxn1* AS4 alternative splicing in the reporter assays. Therefore, nPTB represents a candidate factor for selective regulation of *Nrxn2* AS4 *in vivo* although additional work will be required to explore this.

The severity of *Nrxn1* alternative splicing perturbation differed between brain areas with most significant effects in caudal and less dramatic alterations in forebrain areas. Notably, SLM1 and SLM2 exhibited potent activities toward the *Nrxn1* splice reporters *in vitro* and might compensate for the loss of SAM68 in those neuronal populations. Alternatively, additional RNA-binding proteins might cooperate with SAM68 in the regulation of *Nrxn1* alternative splicing in these cells.

Neural Activity-Dependent Regulation of Neurexins by SAM68

SAM68 and other STAR-family proteins have been established as RNA regulators downstream of growth factor signaling in non-neuronal cells (Matter et al., 2002; Stoss et al., 2004; Valacca et al., 2010). Our work uncovered a novel mode of SAM68 regulation by neuronal activity and CaMKIV and identified serine 20 as a depolarization-dependent phosphorylation site. Intriguingly, SAM68 shares sequence similarity with SF1, a component of the constitutive splicing machinery. In SF1 serine 20 phosphorylation controls interaction with U2AF65 and, thereby, regulates spliceosome assembly (Wang et al., 1999; Selenko et al., 2003). Whether SAM68 phosphorylation at serine 20 functions in an analogous manner remains to be examined, but, notably, SAM68 has been suggested to interact with U2AF65 in non-neuronal cells (Tisserant and König, 2008).

CaMKIV is a major regulator of depolarization-dependent gene expression and alternative splicing but the RNA-binding proteins acting downstream of CaMKIV have remained unknown (Xie and Black, 2001; Xie et al., 2005). Loss of depolarization-dependent *Nrxn1* splicing regulation in SAM68 KO neurons establishes SAM68 as a key mediator of this pathway. Notably, *Nrxn1* exon 20 does not contain recognizable CaRE sequences (unpublished data) and the alternative splicing of several CaRE containing pre-mRNAs was not impaired in SAM68 KO neurons. This suggests that CaMKIV-induced regulation of alternative splicing is relayed via multiple different RNA-binding proteins (or combinations thereof) that bind different recognition elements in their respective target pre-mRNAs. Genome-wide mapping of targets for the neuronal splicing factors NOVA1 and 2 has revealed coordinate regulation of a specific set of

mRNAs encoding synaptic proteins (Ule et al., 2005). Given the significant number of mRNAs that undergo depolarization-dependent alternative splicing, it will be important to map the entire SAM68-dependent program of activity-dependent alternative splicing in the future.

EXPERIMENTAL PROCEDURES

Alternative Splicing Analysis

Oligonucleotide primers used for semiquantitative PCR are listed in Table S1. Input cDNA amounts were titrated to ensure linear relationships between templates and amplification products. DNA fragment intensities were quantified by image analyzer (FAS-III, Toyobo) and ImageGauge software (Fujifilm). Quantitative PCR was performed on a StepOnePlus qPCR system (Applied Biosystems). Gene expression assays (*Nrxn1*:Mm00660298m1, *Gapdh*:Mm99999915 g1, *Khdrbs1*:Mm00516130_m1 Applied Biosystems) or custom-designed assays (Table S2) were used with TaqMan Master Mix (Applied Biosystems) and comparative C_T method. The mRNA levels were normalized to that of *Gapdh* mRNA.

For *in vivo* stimulation by kainate, adult mice (2–3 months old) were anesthetized by intraperitoneal injection of 100 mg/ml ketamine and 10 mg/ml xylazine. A small hole in the occipital bone was made with a dental drill. A 33 gauge microsyringe was inserted into one hemisphere of the cerebellar cortex, and 2–3 μ l of 50 μ M kainic acid in saline containing bromophenol blue dye (0.5 mg/ml) was infused (0.4 μ l/min). Five hours after injection, tissue was dissected and subjected to quantitative PCR analysis. All procedures related to animal experimentation were reviewed and approved by the Kantonales Veterinäramt Basel-Stadt.

IRRE Assay

Splice reporter and EGFP-expression vector (2:1 ratio) were delivered into E14.5 precursor cells of cortical pyramidal cells by *in utero* electroporation with five pulses (50V, 50 ms, each spaced by 950 ms). Dams were brought to term and EGFP-positive cortical tissue was dissected from P6 pups and processed for splicing analysis with primers unique to the reporter sequences.

Neuronal Cell Culture

Cerebellar cultures and mixed culture assays were performed essentially as described (Scheiffele et al., 2000). Briefly, cerebellar neurons (1×10^5 /cm²) were maintained for 9 days *in vitro* and NL- or GluD2-expressing HEK293 cells (2×10^4 /cm²) were added to the culture for an additional 2 days. Cells were fixed and processed for immunohistochemistry and image analysis. For depolarization, the potassium concentration in the medium was elevated by addition of 25 mM KCl from DIV6.

Statistical Analysis

Pairwise comparisons were performed using Student's *t* test. For multiple comparisons, analysis of variance (ANOVA) followed by Bonferroni test was used. Data are represented as the mean \pm standard error of the mean (SEM). Statistical comparison of vGluT1 staining intensity distributions in WT and SAM68 KO cerebellum was done using the Kolmogorov-Smirnov test. Significance is indicated as follows: *** *p* < 0.001, ** *p* < 0.01, * *p* < 0.05.

See Extended Experimental Procedures for further details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2011.11.028.

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